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The genus *Isotomurus*: where molecular markers help to evaluate the importance of morphological characters for the diagnosis of species

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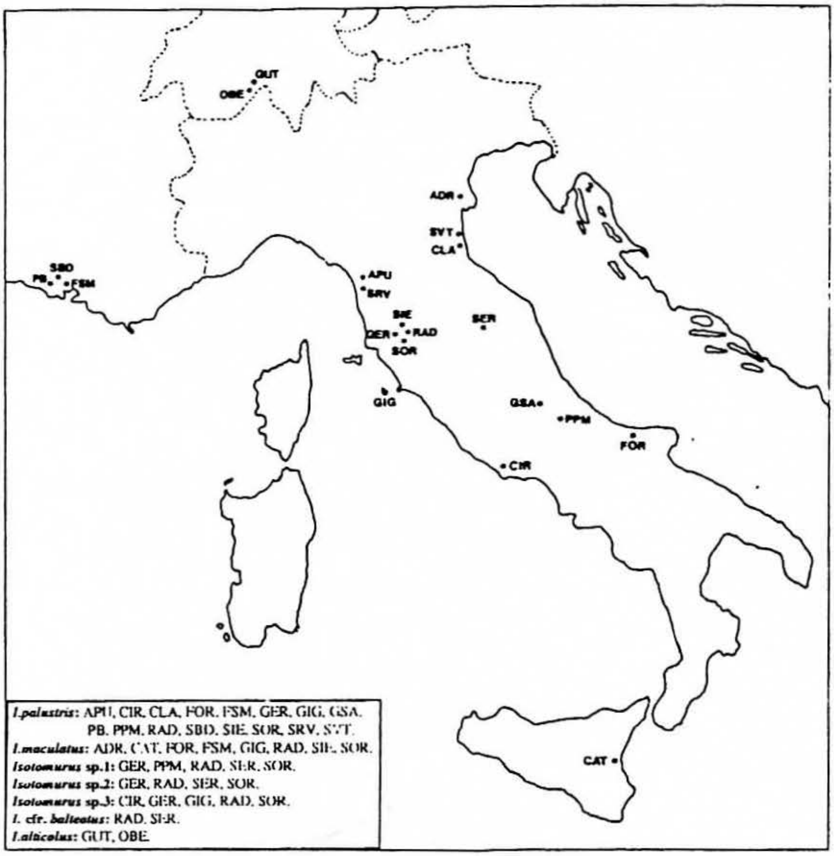
ABSTRACT. Three independent genetic markers (allozymes, mitochondrial and nuclear ribosomal DNA sequences) were studied to evaluate the importance of the few morphological characters (pigmentation, trichobothria, etc.) used to distinguish species of the genus *Isotomurus*. Examples of genetically well differentiated species hidden behind slight morphological differences in pigmentation patterns are reviewed. In addition, molecular data were used to reveal evolutionary relationships within the genus.

Molecular markers have become a fundamental tool in systematics because they provide an independent set of characters, in addition to the classical morphological ones, for species diagnosis and the evaluation of systematic relationships (HILLIS and MORITZ 1990). Besides the well-known allozymes, now widely used for this purpose in most non-microscopic organisms (AVISE 1975; BUTH 1984; FRATI et al. 1989; MURPHY et al. 1990), we recently also used DNA sequences, the collection and analysis of which has been made extremely faster by the development of the technique of the Polymerase Chain Reaction (PCR: SAIKI et al. 1985; MULLIS et al. 1986). PCR allows the amplification of DNA fragments from nuclear and organelle genomes and provides sufficient amounts of DNA for sequencing with conventional methods. The technique is essentially simple (SIMON et al. 1991) and is therefore accessible to researchers with less expertise in molecular biology. The use of universal primers (SIMON et al. 1994), whose sequence conservation allows them to work in a wide variety of taxa, including those for which no previous sequence information exists, makes it possible to obtain this information quite quickly in species at different taxonomic levels. These sequences can then be used to infer evolutionary relationships (AVISE et al. 1987; SIMON et al. 1994). A further useful feature of PCR is that it only requires tiny amounts of starting material, and can be used to amplify DNA from small organisms (like *Collembola*) or even alcohol-preserved and museum specimens (FANG et al. 1993).

In this paper we give several examples of how genetic markers (both allozymes and DNA sequences) can provide information useful for the diagnosis of species and how they can be used to evaluate the diagnostic importance of morphological characters, such as pigmentation and chaetotaxy. The examples are all drawn from the genus *Isotomurus*, especially species of the *I. palustris* group. This genus provides quite a good study material. The characters used for the diagnosis of species, mainly chaetotaxy and pigmentation, are variable and unstable. In the *I. palustris* species group, for example, chaetotaxy appears to be constant and the pattern of pigmentation is the only feature by which species can be discriminated (CASSAGNAU 1987). DEHARVENG and LEK (1993) recently attempted to introduce new morphological characters to elucidate intrageneric taxonomy. They were able to distinguish five different groups of species based on a number of features including abdominal trichobothria, number of bristles S on Abd. V and number of chetae on the tenaculum. Diagnosis of species in the *I. palustris* group, however, is less clear and still based on body colour. Patterns of pigmentation show a great deal of variation within and between geographic populations, with the result that many forms, varieties and subspecies have been erected, but their taxonomic significance is often unclear and controversial (POINSOT-BALAGUER 1972, 1976; CASSAGNAU 1987). Ecomorphosis (CASSAGNAU and LAUGA-REYEL 1987) provides a further element of confusion within the genus.

The complete data set of our study consisted of seven presumed species collected in 21 localities (Fig. 1). Each of the seven presumed species was selected on the basis of a specific pattern of pigmentation, which allowed us to differentiate it from the others. Since pigmentation patterns may differ very slightly and may be variable in a given species, in some cases the diagnosis of the species was possible only after detecting diagnostic loci by allozyme electrophoresis. When distinct


GUT
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PPM
FOR
CIR
CAT
I.palustris: APU, CIR, CLA, FOR, FSM, GER, GIG, GSA, PB, PPM, RAD, SBD, SIE, SOR, SRV, SVT.
I.maculatus: ADR, CAT, FOR, FSM, GIG, RAD, SIE, SOR.
Isotomurus sp.1: GER, PPM, RAD, SER, SOR.
Isotomurus sp.2: GER, RAD, SER, SOR.
Isotomurus sp.3: CIR, GER, GIG, RAD, SOR.
I. cfr. *balteatus*: RAD, SER.
I.alticolus: GUT, OBE

1. Map of collecting localities. Key to populations: ADR: Adria; APU: Apuane; CAT: Catania; CIR: Circeo; CLA: Classe; FSM: Fos sur Mer (F); FOR: Fortore; GER: Gerfalco; GIG: Isola del Giglio; GSA: Gran Sasso; GUT: Guttannen (CH); OBE: Oberaar (CH); PB: Pioch Badet (F); PPM: Pennapiedimonte; RAD: Radi; SDB: Salin de Badon (F); SER: Bocca Serriola; SIE: Siena; SOR: Sorra; SRV: Serravezza; SVT: San Vitale. The distribution of each of the seven species is described in the box

allelic patterns corresponded to sufficiently constant pigmentation patterns, the latter could be used as markers of species differentiation. The seven presumed species included: *I. palustris* and *I. maculatus*, whose specific status was determined in CARAPELLI et al. (1995) and is discussed later; a form whose pigmentation pattern strongly resembles that of *I. balteatus* (but is differentiated from it by the trichobothria, see later), which we call *I.* cfr. *balteatus*; three forms, referred to as *Isotomurus* sp.1, sp.2 and sp.3, which we were not able to assign to any known species. Sp.1 and sp.2 have pigmentation patterns similar to *I. palustris* but sp.1 is constantly characterized by a green background while that of sp.2 is yellowish or greenish and the median longitudinal stripe is usually fainter and interrupted. *Isotomurus* sp.3 is characterized by uniform blue-violet colour, without any particular pattern. The last species was *I. alticolus*, collected in two sites in Switzerland. As shown in Fig. 1, more than one presumed species was present in several localities, with the highest number found at RAD (six). A total of 42 populations were, therefore, scored.

Genetic markers were taken from three independent data sets, including a survey of 12 enzyme loci for the 42 populations, the sequences of the mitochondrial COII gene (672 base pairs in seven populations of three presumed species) and the sequences of the nuclear ribosomal D3 fragment (276 base pairs in ten populations of six presumed species).

I. PALUSTRIS VS. *I. MACULATUS*

At the beginning of our work on *Isotomurus*, the first point we examined was the taxonomic status of some of the varieties (or subspecies according to different Authors) of the species *I. palustris*, especially the form *maculata* (sensu CASSAGNAU 1987), which POINSOT-BALAGUER (1972) described as a subspecies named *I. palustris palustris*. We collected these two forms sympatrically in five localities from southern France to southern Italy and the results, extensively discussed in CARAPELLI et al. (1995), clearly showed that they must be regarded as two different species.

In each locality, a number of enzyme loci (from 3/12 to 7/12) were fixed for alternative allele patterns and the absence of hybrids demonstrated unequivocally that the two species do not cross. Even though it is not possible now to decide what kind of mechanism of reproductive isolation is occurring (whether pre- or post-zygotic, ecological or else), its presence is unquestionable. DNA data showed that the two species diverge by 17% in their mtCOII nucleotide sequence, but only little variation is present within them. We concluded that, although the different patterns of pigmentation show intraspecific variation, they can be considered markers of species differentiation. This suggests that the same might be true for other forms or subspecies within the genus.

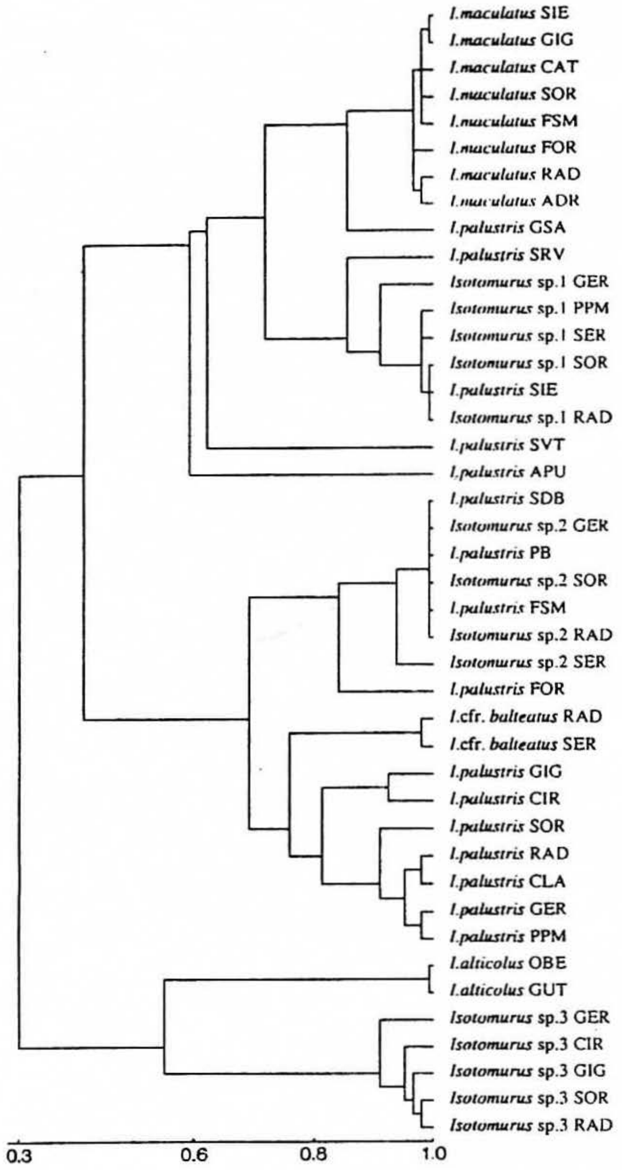
We then compared our specimens of *I. maculatus* with those originally collected and described by Dr. SCHÄFFER at the end of the last century (SCHÄFFER 1896), and kept at the Zoological Museum of Hamburg (Germany). The pigmentation patterns of the Italian and

German samples were quite similar, confirming that our specimens are correctly assigned to the species *I. maculatus*. Our future approach will be to collect samples of this species in SCHÄFFER's original sampling site (around Hamburg) and compare them with southern populations, using selected genetic markers to evaluate the homogeneity of the species along a north-south axis.

VARIABILITY WITHIN *I.PALUSTRIS*

Allozyme data suggested that all the populations of *I. maculatus* are quite homogeneous. The UPGMA dendrogram (SNEATH and SOKAL 1973; Fig. 2), drawn on the basis of genetic similarity calculated from allelic frequencies (ROGERS 1972), illustrates the phenetic relationships between the populations studied and shows all the populations of *I. maculatus* clustered together. The same cannot be said for the populations which we determined as *I. palustris* on the basis of pigmentation patterns. They are scattered throughout the dendrogram reflecting genetic similarity values (ROGERS 1972) as low as 0.151 and genetic distance values (NEI 1978) as high as 2.465. They can be roughly divided into three main clusters. One, containing the populations from SIE and SRV, is grouped with the populations determined as *Isotomurus* sp.1. A second, including most of the *I. palustris* populations, also contains the two populations whose pigmentation pattern resembles *I. balteatus* (the problem of those specimens will be discussed later). A third group, comprising the three French populations and the one from FOR, includes all the populations determined as *Isotomurus* sp.2. Moreover, three populations (GSA, SVT and APU) diverge somewhat from all the others, with GSA apparently more similar to *I. maculatus*. This differentiation, which does not follow any clear geographic pattern, suggests that more than one species may be hidden under the general pigmentation pattern of *I. palustris*. If this is true, we will have to examine pigmentation patterns more carefully looking for possible diagnostic features. It must be remembered, in this respect, that genetic divergence between allopatric populations is not necessarily due to effective species differentiation, but may also be caused by prolonged geographic isolation and genetic drift. The real *I. palustris* might even be one of our sp.1 or sp.2, but we need to compare these and additional populations with material collected in the site of the original description by MÜLLER (1776).

Although DNA data were only available for a subset of populations, it confirmed the heterogeneity of the specimens determined as *I. palustris*, and in particular the affinity between *I. palustris* from SIE and *Isotomurus* sp.1. In the mtCOII gene (672 bp) the five populations



2. UPGMA dendrogram constructed on the basis of ROGERS (1972) genetic similarity calculated on allozyme frequencies at 12 loci

of *I. palustris* tested diverged by as much as 22% of their nucleotide sequence, while *I. palustris* from SIE had only 6 nucleotide substitutions (1%) with respect to *Isotomurus* sp.1 from RAD. This is reflected in the UPGMA dendrogram of Fig. 3, where the close affinity between the populations from RAD and GER (with only 1.9% of sequence divergence) is also evident.

In conclusion, genetic divergence among populations determined as *I. palustris* seems to exist without substantial morphological differentiation. Less evident differences in pigmentation patterns (or other morphological characters) probably need to be found in order to discriminate new species; genetic markers are a valuable tool to guide this investigation. Additional genetic information, however, is necessary to elucidate the systematic position of such populations as GSA, SVT and APU which is not apparently explained by any reasonable hypothesis.

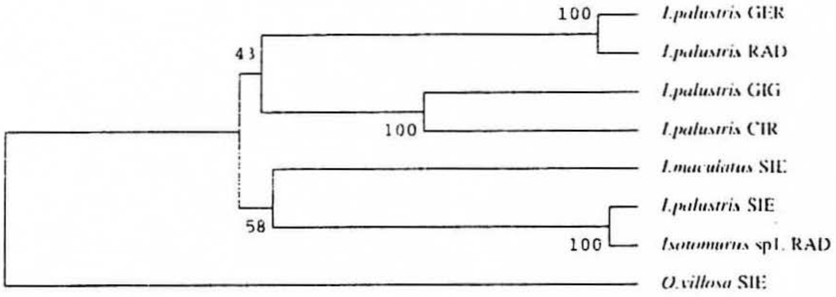
SIX SYMPATRIC SPECIES IN RAD

The genus *Isotomurus* is indeed a species-rich genus. According to genetic markers, there is a strong differentiation between sympatric species. In one of our sampling sites, Radi (RAD), six different species were found in the same place. Their species status is demonstrated by the presence of loci fixed for alternative alleles (Table 1). Fig. 4 shows the phylogenetic relationships between the six species as inferred from the sequences of the D3 fragment. The tree, constructed with the principle of maximum parsimony, does not completely agree with the relationships between the same populations shown in Fig. 2, although some groups, like *I. palustris* + *I.* cfr. *balteatus* and *Isotomurus* sp.1 + *I. maculatus*, are present in both. Additional data are needed and we are sequencing the COII gene for these six species in order to shed some light on this problem.

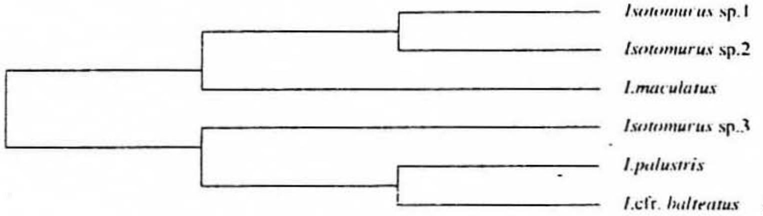
Table 1

Number of loci fixed for alternative alleles (below the diagonal) and NEI's (1978) genetic distance values between the six presumed species found in Radi

	Species	1	2	3	4	5	6
1	*I.palustris*	—	.795	1.293	.412	1.072	.350
2	*I.maculatus*	5/12	—	.575	.990	1.251	.608
3	*Isotomurus* sp.1	7/12	4/12	—	1.992	1.215	1.124
4	*Isotomurus* sp.2	3/12	6/12	8/12	—	1.431	.889
5	*Isotomurus* sp.3	5/12	6/12	6/12	6/12	—	1.290
6	*I.* cfr. *balteatus*	2/12	4/12	6/12	5/12	6/12	—



3. UPGMA dendrogram based on the mtCOII sequence (672 bp). Bootstrap values are indicated at the nodes. The sequence of *Orchesella villosa* has been used as outgroup



4. Phylogenetic relationships among the six presumed species found in Radi. The tree is drawn upon the principle of maximum parsimony and is based on the D3 fragment sequence (276 bp)

It is difficult to reconstruct the history of the whole genus and to understand the reason for the presence of so many species. Speciation might have been caused by geographic isolation due to land fragmentation (for instance in the Italian peninsula before and during glacial periods), but it may also have been driven by ecological preferences. Ecological differentiation is a possibility in RAD, as certain species seem to prefer (but are not limited to) particular microhabitats in the same area. A more detailed study needs to be performed to evaluate the importance of these ecological characteristics in relation to the speciation process.

THE PROBLEM OF *I. CFR. BALTEATUS*

In the examples illustrated so far, we have seen that differences in pigmentation patterns are usually backed by genetic differentiation and probably indicate species differentiation. Genetic markers help to bring out these differences and suggest that sibling species might be present, especially among the specimens determined as *I. palustris*. We found an example, however, in which different morphological characters, namely pigmentation and trichobothria, give contrasting information. In two sites from central Italy, RAD and BSR, we collected specimens (which we refer to as *I.* cfr. *balteatus*) with a pigmentation pattern identical to *I. balteatus*. However, the number of abdominal trichobothria is clearly different. In these Italian populations, in fact, it is 3+3 in Abd. II, 3+3 in Abd. III and 1+1 in Abd. IV (formula: 3+3+1), whereas *I. balteatus* from France is described as having 0+0+1 or 0+1+1 trichobothria (POINSOT-BALAGUER and FERARD 1983; DEHARVENG and LEK 1993). A similar situation was observed by YOSHII and SUHARDJONO (1992) in specimens from Timor island (with 3+3+1 trichobothria) and they were given the name of *I. tricuspis*. In addition, the Italian specimens are much larger than the French ones. They seem to belong to different species which have evolved the same patterns of pigmentation. Genetic markers will hopefully help to decide the status of these Italian populations and our future project will be to collect specimens of the "true" *I. balteatus* and compare them by means of allozymes and DNA sequences.

FINAL REMARKS

The correspondence between pigmentation patterns and genetic markers found in many species of *Isotomurus* suggests that this genus comprises so-called "colour pattern species", a term introduced by YOSHII (1989) who noted how, in many genera of Entomobryomorpha (like *Oceaniella*, *Homidia* and *Salina*), species can be identified only on the basis of the pattern of pigmentation. This concept is only temporary, as further and more accurate studies may detect new morphological features which can be used to distinguish different species. In genera like *Entomobrya*, *Seira* and *Callyntrura* species diagnosis was based only on pigmentation patterns until new morphological characters were found. The same might be true for the species of *Isotomurus* (YOSHII 1963; DA GAMA 1964). Additional morphological studies (like the one by DEHARVENG and LEK 1993) may be needed to find diagnostic characters. To complicate this picture, there appear to be cases in which the same pigmentation pattern is shown by species clearly differentiated by other morphological characters; our specimens of *I.* cfr. *balteatus* are an example. Similar pigmentation patterns may have adaptive value, and therefore be due to convergent evolution, but this is not easy to demonstrate. It is true, however, that the same patterns can sometimes be observed in species of different genera (POTAPOV, in prep.).

There seem to be more doubts than certainties in the systematics of the genus *Isotomurus*. This situation deserves a careful study because it could have implications for other genera of *Collembola* in which pigmentation patterns are the only significant character for distinguishing species.

In conclusion, genetic markers have shown that the number of intrageneric speciation events is unexpectedly high in many organisms, particularly insects. Therefore they appear to be a fundamental tool also in collembolan systematics, especially when diagnostic characters are lacking or difficult to study and intraspecific and intrapopulation variability is high.

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